

In Vitro Thymosin Effect on T Lymphocytes in Alcoholic Liver Disease

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The *in vitro* effect of thymosin fraction 5, a thymic gland extract, on thymus-dependent lymphocytes was studied in 12 patients with alcoholic hepatitis (AH) and in 18 patients with compensated alcoholic cirrhosis (CAC). The baseline number and proportion of spontaneous rosette-forming T lymphocytes were significantly reduced in AH as compared to the controls. No such decrease was seen in CAC. When the lymphocytes of patients with AH were incubated in the presence of thymosin fraction 5, the subsequent number and proportion of rosette-forming T lymphocytes showed a significant increase. There was no significant response to thymosin in CAC or in control subjects. There appears to be a subset of immature T lymphocytes in patients with AH that is responsive to exogenous thymic factors with subsequent activation of the capacity to form spontaneous rosettes.

INTRODUCTION

Identification and enumeration of peripheral T and B lymphocytes in patients with alcoholic liver disease has resulted in reports of diminished numbers of T but not B cells (1-8). Additional alterations in cell-mediated immunity (1, 5) suggest the possibility of a dysfunction in the regulatory role for the endocrine thymus in the ontogenesis and function of the T-lymphoid system.

Thymosin, a polypeptide extract of calf thymus, has been shown, *in vitro*, to increase the percentage of spontaneous T-cell rosettes (ER) in patients with a variety of primary and secondary immunodeficiency diseases, but not in most normal individuals (9-11). Thymosin is believed to act by inducing maturation of undifferentiated lymphocytes predetermined to follow the T-cell pathway (12). The Wara-Ammann ER assay (13) measures the *in vitro* response of ER in the presence or absence of thymosin. Patients whose ER are increased following incubation with thymosin appear to have a greater than normal number of precursor T cells and a thymosin-dependent immunodeficiency disorder. Failure to increase ER with thymosin may indicate that there is an adequate endogenous level of thymosin or a thymosin-independent disorder.

Determination of ER formed under conditions where the sheep red blood cell (SRBC) lymphocyte ratio exceeds 32/1 may not accurately reflect cellular immune competency, but instead may be an indicator of the presence and distribution of the T cell (14). Recent studies indicate that enumeration of ER in a system designed to measure the percentage of "avid" rosettes provides a more sensitive index for the determination of an altered cellular immune state (7, 15). The "avid" rosette test utilizes a SRBC/lymphocyte ratio of 8/1 which identifies T cells that

demonstrate an avidity for SRBC. This avidity may be related to the density of SRBC receptors on the surface of T cells and/or the affinity of these receptor sites for SRBC (15). In addition, the "avid" rosette may be comparable to the "active" rosette previously described (16). The "active" ER test also utilizes a SRBC/lymphocyte ratio of 8/1, but unlike the "avid" ER assay, includes fetal calf serum in the media. In addition, the ER count is initiated approximately 5 min following addition of the SRBC to the lymphocyte suspension, while in the "avid" ER test, the SRBC/lymphocyte suspension is incubated overnight at 4°C. Chisholm and Tubergen (15) have suggested that the SRBC/lymphocyte ratio is the critical factor in the formation of ER rather than the time duration or temperature of incubation. Accordingly, avid ER and B lymphocytes were enumerated in patients with alcoholic liver disease; the effect of preincubation with thymosin on avid ER formation was also evaluated in such patients and controls. Our results show that thymosin increased avid ER in patients with alcoholic hepatitis (AH) but not in patients with compensated alcoholic cirrhosis (CAC) or in age-equivalent controls. This suggests that there is a thymosin-dependent immunodeficiency state in AH which appears to be reversible with resolution of the acute episode.

MATERIALS AND METHODS

Study groups. Thirty male patients with alcoholic liver disease were studied. Twelve patients were considered to have AH based on clinical and laboratory findings which included the following criteria determined after at least 5 days in the hospital: A recent history of alcohol consumption in excess of 1 pint of whiskey per day or its alcoholic equivalent; hepatomegaly on physical examination and/or liver scan, a total bilirubin greater than 4 mg%; and abnormalities in at least two of the following: (a) serum glutamic oxaloacetic transaminase (SGOT) more than 80 IU/ml (normal range 5–25 IU), (b) a serum albumin of less than 3 g%, or (c) a prothrombin time more than 2 sec over the control value (Table 1). Coagulation deficiencies and/or the presence of marked ascites precluded liver biopsy in all but five of the patients studied. In all cases where biopsy was possible, the clinical diagnosis of AH was confirmed by the presence of alcoholic hyalin, liver cell necrosis, polymorphonuclear neutrophil leukocyte (PMN) infiltration. Four of the five biopsies revealed concomitant micronodular cirrhosis. These criteria for the diagnosis of AH are similar to those used in a previous study of corticosteroid therapy in alcoholic hepatitis (17). In addition, none of the subjects manifested

TABLE I
LIVER FUNCTION PROFILE OF PATIENT GROUPS^a

Group	Age	Total serum bilirubin (mg/100 ml)	Serum alkaline phosphatase (IU)	SGOT (IU)	Serum albumin (g/100 ml)	Prothrombin time (%)
Alcoholic hepatitis	50.0 ± 2.0	9.9 ± 2.9	219 ± 32	135 ± 24	2.6 ± 0.1	59.0 ± 6.7
Compensated alcoholic cirrhosis	51.0 ± 2.4	1.8 ± 0.4	144 ± 18	70 ± 13	3.5 ± 0.2	71.4 ± 4.4

^a Values shown are means ± SEM.

evidence of a bacterial or fungal infection, acute renal impairment, or significant upper gastrointestinal bleeding at the time of evaluation.

Eighteen alcoholic patients were diagnosed as having CAC on the basis of biopsy-proved micronodular cirrhosis without superimposed hepatitis, with known previous heavy alcohol intake and demonstrated clinical and laboratory findings compatible with quiescent disease (Table 1).

Control subjects consisted of 30 healthy volunteers not known to have liver disease. In addition, none manifested evidence of an acute illness. Controls or patients with malignancy, with a history of recent viral upper respiratory illness, or recipients of blood transfusions within 4 weeks of the examination were excluded. No patients were receiving steroids or other immunosuppressive therapy.

Two methods of evaluation were used. The first compared the study group(s) with age-equivalent controls and the second compared the study group(s) with a larger, total control group.

Separation of peripheral lymphocytes. Venous blood was drawn into a 50-ml syringe containing 50 units/ml of preservative-free heparin. The blood was diluted 1/1 in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) to which 0.35 g of sodium bicarbonate was added for each liter of solution. The diluted blood was layered in 10-ml aliquots onto 3 ml of Ficoll-Hypaque discontinuous gradient and spun at 400g for 30 min at 20°C. The cells at the interface were removed, washed three times in HBSS, and adjusted to a final concentration of 5×10^6 cells/ml. A Wright's stain smear revealed 90-95% lymphocytes with the remaining cells identified as monocytes. No attempt was made to remove the latter cells.

ER and thymosin treatment. Purified bovine thymosin fraction 5 (clinical quality) was prepared (18) in concentrations of 400, 200, and 100 $\mu\text{g/ml}$ in 0.9 N NaCl. Thymosin fraction 5 effect was determined as outlined by Wara and Ammann (13) with the single alteration that the response in the number of avid ER, rather than total ER, was determined. Briefly, 0.1 ml of the lymphocyte suspension was added to 0.1 ml of HBSS and in other tubes, to 0.1 ml of the three concentrations of thymosin fraction 5. Triplicate samples were prepared. The cell suspensions were incubated at 37°C for 10 min. SRBC from the same animal were preserved in Alsever's solution and were always less than 3 weeks old. The SRBC were washed three times in HBSS and resuspended at 2×10^7 cells/ml. SRBC suspension, 0.2 ml, was added to each of the tubes with a final SRBC/lymphocyte ratio of 8/1. The tubes were capped and centrifuged for 5 min at 180g and allowed to sit overnight (18-24 hr) at 4°C. The cell pellet was then resuspended by gently rotating at 180 rpm on a platform for 4 min. Lymphocytes binding more than three red cells were scored as avid T cells (7). The total ER count was obtained by incubating the lymphocytes at a saturated SRBC/lymphocyte ratio of 60/1, in the absence of thymosin. All tubes were counted in blind fashion with respect to patient source and knowledge of the presence of thymosin fraction 5 or the control situation. The mean percentage of ER-forming cells and the SEM were determined in the control and patient samples at that concentration of thymosin which produced the highest ER formation. Student's independent *t* test was used to

compare study groups. Student's dependent *t* test was used to compare ER in the presence or absence of thymosin within the same study groups.

B-lymphocyte assay. The B-lymphocyte count was determined by the percentage of lymphocytes staining with fluoresceinated goat antiserum to human immunoglobulin (Ig). Briefly, 0.1 ml of the lymphocyte suspension was incubated with 0.02 ml of an 1/8 dilution of polyvalent goat antiserum against human immunoglobulin in veronal buffer solution (VBS) supplemented with 10% fetal calf serum for 20 min at 37°C. The cells were washed twice with VBS and the percentage of cells with membrane-bound immunoglobulin counted using a Zeiss fluorescent microscope.

Recently, concern has been raised that the standard fluorescein-labeled anti-human Ig used in a number of investigations resulted in an over estimation of the number of B cells (19). It has been shown that rabbit anti-human Ig may reaggregate after ultracentrifugation, or form immune complexes and then attach to the Fc receptors of non-B lymphocytes. Thus, it was suggested that F(ab')₂ fragments of rabbit anti-Ig should be used instead. However, recent reports indicate that whole goat anti-human Ig may be used in place of F(ab')₂ fragments (20).

RESULTS

As shown in Table 2, the total white blood cell (WBC) as well as the PMN counts were significantly higher in patients with AH than in the controls. No differences were noted between patients with CAC and controls. The proportion and absolute lymphocyte counts were significantly lower in AH than in the controls, while no such differences were noted between patients with CAC and the controls.

Avid ER

There was a highly significant decrease in both the proportion and absolute number of avid ER in patients with AH ($P < 0.001$). In contrast, the proportion and number of avid ER were similar for patients with CAC and the controls (Table 3).

B Lymphocytes

There were no differences in the proportion of B cells in any of the groups. The absolute number of B cells was diminished in AH and probably was a reflection of the decreased peripheral lymphocyte count in AH (Table 3). The proportions of B cells obtained in the patient and control groups is higher than the currently accepted figures of 4–10%. This discrepancy is probably attributable to the inclusion in our count of monocytes with attached cytophilic IgG antibody (20).

Thymosin Effect

Addition of thymosin to lymphocytes prior to incubation with SRBC resulted in significant increases in avid ER in patients with AH (Table 4). No significant changes were noted in patients with CAC or in any of the control groups.

DISCUSSION

A number of studies have reported diminished peripheral blood T lymphocytes in alcoholic liver disease in systems designed to enumerate "total" T cells (1–6). As shown in Table 5, patients with AH had a significantly lower proportion and

TABLE 2
PERIPHERAL WHITE BLOOD CELL POPULATIONS^a

Study group	Number	White blood cells		PMN		Lymphocytes	
		No./mm ³	No./mm ³	Percentage	Absolute No./mm ³	Percentage	Absolute No./mm ³
Alcoholic hepatitis	12	8992 ± 959 ^b	8992 ± 912 ^d	75.1 ± 3.9 ^c	6992 ± 912 ^d	21.3 ± 3.2 ^e	1723 ± 206 ^f
Age-equivalent control	12	7542 ± 530	4632 ± 431	61.0 ± 3.5	4632 ± 431	34.8 ± 3.0	2604 ± 295
Compensated alcoholic cirrhosis	18	7294 ± 950	4396 ± 666	58.0 ± 3.2	4396 ± 666	35.3 ± 3.1	2392 ± 277
Age-equivalent control	18	7661 ± 395	4796 ± 330	62.5 ± 3.0	4796 ± 330	33.6 ± 2.5	2575 ± 249
Total control	30	6890 ± 325	4239 ± 253	61.2 ± 1.9	4239 ± 253	33.8 ± 1.7	2329 ± 170

^a Means ± SEM.

^b $P < 0.05$, total control.

^c $P < 0.005$, total control; $P < 0.02$, equivalent control.

^d $P < 0.001$, total control; $P < 0.05$, equivalent control.

^e $P < 0.001$, total control; $P < 0.005$, equivalent control.

^f $P < 0.05$, total control and equivalent control.

TABLE 3
PERIPHERAL AVID ER AND B CELLS IN ALCOHOLIC LIVER DISEASE^a

Group	Number	Age	Avid T lymphocytes			B lymphocytes		
			Percentage	Mean No./mm ³	Mean No./mm ³	Percentage	Mean No./mm ³	
Alcoholic hepatitis	12	50.0 ± 2.0	16.0 ± 1.1 ^b	280 ± 40 ^b	18.8 ± 1.2	291 ± 42 ^c		
Equivalent control	12	50.7 ± 2.0	25.7 ± 1.8	682 ± 94	18.7 ± 1.6	453 ± 35		
Compensated alcoholic cirrhosis	18	51.0 ± 2.4 ^d	24.5 ± 1.1	588 ± 81	18.9 ± 1.2	477 ± 68		
Equivalent control	18	50.5 ± 2.9	25.3 ± 1.2	652 ± 69	17.4 ± 1.3	423 ± 36		
Total control	30	43.5 ± 2.5	25.5 ± 0.9	593 ± 46	18.9 ± 1.0	425 ± 26		

^a Means ± SEM.

^b $P < 0.001$, equivalent and total control.

^c $P < 0.01$, equivalent control; $P < 0.02$, total control in 10 patients evaluated.

^d $P < 0.05$, total control.

TABLE 4
EFFECT OF THYMOSIN ON AVID ER^a

Group	Baseline avid T-cell rosettes		Avid T-cell rosettes after thymosin	
	Percentage	Mean No./mm ³	Percentage	Mean No./mm ³
Alcoholic hepatitis	16.0 ± 1.1 ^b	280 ± 40 ^b	20.7 ± 1.2 ^c	365 ± 52 ^d
Equivalent control	25.7 ± 1.8	682 ± 94	27.6 ± 1.8	712 ± 85
Compensated alcoholic cirrhosis	24.5 ± 1.1	588 ± 81	25.4 ± 1.0	608 ± 79
Equivalent control	25.3 ± 1.2	652 ± 69	26.5 ± 1.3	672 ± 64
Total controls	25.5 ± 0.9	593 ± 46	26.2 ± 1.0	604 ± 45

^a Mean ± SEM.

^b $P < 0.001$, equivalent and total control.

^c $P < 0.001$, AH baseline, Student's paired t test.

^d $P < 0.005$, AH baseline, Student's paired t test.

count of "total" T cells than the controls while a similar evaluation of patients with CAC did not reveal significant differences.

The observation of normal avid ER and total T-cell counts in CAC is supported by the work of some investigators (5) and is in disagreement with others (2-4). These conflicting results may represent differences in patient selection as well as criteria for exclusion from the study. As an example, the requirement that subjects

TABLE 5
"TOTAL" T-CELL ROSETTES^a

Group	Percentage ER	Mean No./mm ³
Alcoholic hepatitis (12)	53.3 ± 1.7 ^b	985 ± 111 ^c
Equivalent control (12)	61.3 ± 1.8	1635 ± 212
Compensated alcoholic cirrhosis (18)	60.6 ± 1.2	1384 ± 190
Equivalent control (18)	62.4 ± 1.3	1625 ± 165
Total controls (30)	61.0 ± 1.1	1437 ± 115

^a Means ± SEM.

^b $P < 0.005$, equivalent control; $P < 0.001$, total controls.

^c $P < 0.05$, equivalent and total controls.

not have a recent upper respiratory illness is predicated on the reported inhibiting effect that the influenza virus has on ER formation (21). Thus, a history of recent upper respiratory illnesses in patients and controls was cause for exclusion from the study.

There are a number of possibilities which may account for the decrease in peripheral T cells in AH. Sensitized lymphocytes may migrate to the liver depleting the peripheral blood of mature T cells (4). T cells may be sequestered in the liver or subjected to the toxic effect of alcohol which could affect cellular function or kinetics (2). In addition, alcoholic hyalin appears to induce complex formation by combining with antibody and is carried as complexes on lymphocytes (22). It is possible such complexes may adhere to the surface of T lymphocytes and prevent rosette formation. A similar mechanism has been suggested to account for the decreased proportion of rosettes seen in systemic lupus erythematosus (11).

A serum factor designated as a rosette-inhibiting factor (RIF) has been described in patients with acute and chronic viral hepatitis (23). RIF has been characterized as an immunoregulatory plasma lipoprotein that can suppress rosette formation of normal T lymphocytes *in vitro*. Lymphocytes affected by RIF can regenerate normal rosette formation when cultured in the presence of normal serum.

Of import to the present study is the observation that RIF inhibits the rosette function of only a subpopulation of T lymphocytes (24). No greater than a 40–50% relative inhibition of rosette function was observed even with a 1000-fold excess of RIF. The number of "active" rosettes is normal however in patients who are RIF (+), while the total number of rosettes is reduced (25). Although a RIF-like factor has not been described in the sera of patients with alcoholic liver disease, its presence would not appear to alter avid rosette counts as this assay appears to identify a subgroup of lymphocytes similar to that of the "active" rosette assay (15).

Alterations in cell-mediated immunity have been reported in alcoholic liver disease. Patients with alcoholic cirrhosis show a decreased ability to be sensitized to dinitrochlorobenzene (1). Production of migration-inhibition factor and fibrogenic factor was increased in lymphocytes of patients with AH incubated with purified alcoholic hyalin but not in healthy controls, acute viral hepatitis, fatty liver or inactive cirrhosis (26, 27). Lymphocytes from patients with AH were found to be cytotoxic to Chang cells (22), autologous liver cells (22), and rabbit hepatocytes (28).

It has been suggested that there is a reversible depression of the cell-mediated immune system in patients with AH, as manifested by cutaneous delayed hypersensitivity and T-cell proportions (5). With recovery from AH, even in the presence of cirrhosis, the T-cell proportions return to the normal range and skin tests for common antigens reveal greater "positivity" when expressed in millimeters of induration (5).

The uniform increase in avid ER in AH after incubation with thymosin (Fig. 1) suggests that there is a subset of lymphocytes that may be considered precursors to cells capable of forming spontaneous rosettes with SRBC. It is believed that immature T lymphocytes may constitute a subset of null cells (10). Recent studies have shown that thymosin induces null cells to form ER in patients with T-cell

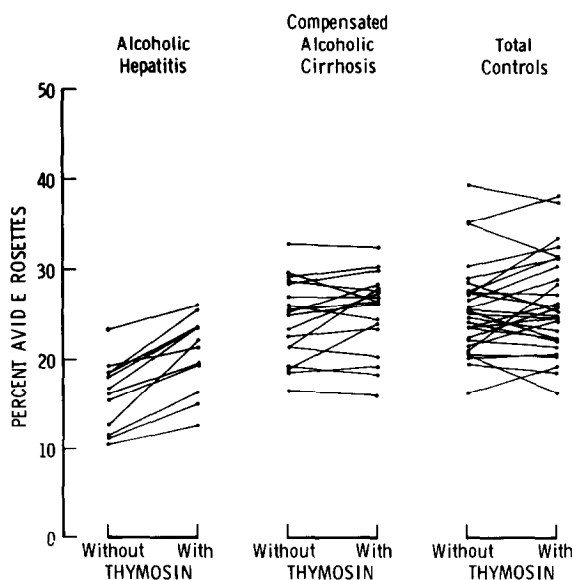


FIG. 1. *In vitro* effect of thymosin on peripheral avid T rosettes in alcoholic liver disease.

defects (29). Moreover, thymosin acts on human null cells possessing T-lymphocyte antigens, but not B-cell antigens (30).

The actual percentage increase in avid ER in AH was small. Thus, while the baseline avid ER in AH was 63% of the control value, thymosin treatment resulted in avid ER attaining 81% of the baseline values for the controls. This may indicate that only a small proportion of lymphocytes induced to maturity by thymosin possess the capability for forming avid ER. It has been shown that the functional change induced by thymosin may not be correlated to total ER formation, but rather, to increased active ER formation (29). No attempt was made to characterize the response of lymphocytes to thymosin in a saturated SRBC/lymphocyte milieu.

The thymus has been shown to exert an endocrine influence on the maturation and function of the immune system. This has been demonstrated previously in a variety of animal and human models by use of thymus gland transplantation and by the administration of thymic extracts (31–33). Thymosin appears to hasten maturation of immature T cells with induction of surface markers found in mature cells (9). The short period of incubation required to induce the appearance of T-lymphocyte surface markers points to a membrane effect rather than activation of nuclear or cytoplasmic mechanisms.

Thymosin increases intracellular levels of cyclic GMP, but not cyclic AMP in murine thymocytes. This finding suggests that a mechanism of action for thymosin may involve modulation of cyclic GMP levels in lymphocytes (34).

It would be premature to ascribe the decreased number of avid ER in AH to dysfunction of the thymic gland, or to alterations in the effective serum thymic factor concentration. It does appear, however, that the target immature T cell retains the capacity to respond to thymic factors.

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